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Advances in Brief

IAP-Family Protein Survivin Inhibits Caspase Activity and Apoptosis Induced by Fas (CD95), Bax, Caspases, and Anticancer Drugs[1]

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Abstract

Survivin is a member of the inhibitor of apoptosis protein (IAP) family. We investigated the antiapoptotic mechanism of Survivin, as well as its expression in 60 human tumor cell lines used for the National Cancer Institute's anticancer drug screening program. In cotransfection experiments, cell death induced by Bax or Fas (CD 95) was partially inhibited (mean ± SD, 65% ± 8%) by Survivin, whereas XIAP, another IAP family member, almost completely blocked cell death (93% ± 4%) under the same conditions. Survivin and XIAP also protected 293 cells from apoptosis induced by overexpression of procaspase-3 and -7 and inhibited the processing of these zymogens into active caspases. *In vitro* binding experiments indicated that, like other IAP-family proteins, Survivin binds specifically to the terminal effector cell death proteases, caspase-3 and -7, but not to the proximal initiator protease caspase-8. Using a cell-free system in which cytosolic extracts were derived from control- or Survivin-transfected cells and where caspases were activated either by addition of cytochrome *c* and dATP or by adding recombinant active caspase-8, Survivin was able to substantially reduce caspase activity, as measured by cleavage of a tetrapeptide substrate, AspGluValAsp-aminofluorocoumarin. Similar results were obtained in intact cells when Survivin was overexpressed by gene transfection and caspase activation was induced by the anticancer drug etoposide. Survivin was expressed in all 60 cancer cell lines analyzed, with highest levels in breast and lung cancers and lowest levels in renal cancers. These findings indicate that Survivin, which is commonly expressed in human tumor cell lines, can bind the effector cell death proteases caspase-3 and -7 *in vitro* and inhibits caspase activity and cell death in cells exposed to diverse apoptotic stimuli. Although quantitative differences may exist, these observations suggest commonality in the mechanisms used by IAP-family proteins to suppress apoptosis.

Introduction

Suppression of apoptosis contributes to carcinogenesis by several mechanisms, including aberrantly prolonging the cell life span, thus facilitating the accumulation of gene mutations, permitting growth factor-independent cell survival, promoting resistance to immune-based cytotoxicity, and allowing disobeyance of cell cycle checkpoints that would normally induce apoptosis (1–3). Defects in apoptotic mechanisms also play an important role in resistance to chemotherapy and radiation (1).

Survivin is a recently described member of the IAP[3] family of antiapoptotic proteins, which are conserved across evolution with homologues found in both vertebrate and invertebrate animal species (4). The baculovirus IAPs, Cp-IAP and Op-IAP, were the first members of this family to be identified based on their ability to functionally complement defects in the cell death inhibitor p35, a baculovirus protein that binds to and inhibits caspases (5, 6). Subsequently, five additional human (XIAP, c-IAP1, c-IAP2, NAIP, and Survivin) and two *Drosophila* homologues have been identified, which have been demonstrated to inhibit cell death (4, 7–12). A central role for IAP-family proteins in programmed cell death regulation in *Drosophila* has been suggested by the finding that several apoptosis-inducing proteins in flies, including reaper, hid, and grim bind to IAPs as part of their cytotoxic mechanism (13, 14). Although the biochemical mechanism by which IAP-family proteins suppress apoptosis has been debated, the human IAPs (XIAP, cIAP1, and cIAP2) have been reported to bind and potently inhibit caspase-3 and -7, with Kis in the range of 0.2–10 nM (15, 16). These caspases operate in the distal portions of apoptotic protease cascades, functioning as effectors rather than initiators of apoptosis (17, 18).

The common structural feature of all IAP family members is a ~70 amino acid motif termed BIR, which is present in one to three copies. Using a mutagenesis approach, we previously showed that the second of the three BIR domains (BIR2) of XIAP is necessary and sufficient for inhibiting certain caspase-family cell death proteases (19), implying that a single BIR domain can possess antiapoptotic activity. Survivin contains a single BIR domain that shares amino acid sequence similarity to the BIR2 region of XIAP (4, 20).

The Survivin protein is abundantly expressed during fetal development in humans, but rarely present in adult tissues (4). However, expression of Survivin has been reported in most human tumors, suggesting that alterations in *Survivin* gene regulation occur commonly during tumorigenesis (4). Overexpression of Survivin in a lymphokine-dependent hematopoetic cell line has been reported to delay cell death induced by factor withdrawal (4). Conversely, antisense-mediated suppression of Survivin expression induces apoptosis in HeLa cells (21).

To explore the mechanism of Survivin function, we compared it with another IAP-family protein, XIAP, with respect to caspase inhibition and suppression of apoptosis induced by stimuli that engage apoptotic pathways at different sites.

Materials and Methods

Cloning of Survivin cDNA, Plasmid Construction, Expression, and Purification of Proteins. A human Survivin cDNA was obtained by reverse transcription-PCR of RNA derived from Jurkat T cells with the following primers based on GenBank accession number U75285 (forward, 5′-GGGAATTCATGGGTGCCCCGACGTTGCC-3′; reverse 5′-CTCTCGAGTCAATCCATGGCAGCCAGCT-3′). PCR products were digested with *Eco*RI and *Xho*I and ligated into pcDNA3myc. Plasmids encoding XIAP, Bax, and Fas have been described previously (15, 22, 23). Recombinant caspase proteins containing His_6 tags were prepared as described previously and were generously provided by G. Salvesen (The Burnham Institute, La Jolla, CA; 24–27).

Transfection of Cultured Cells. Subconfluent 293 cells were transfected in 6-cm dishes using a calcium phosphate method with 1 μg of pcDNA3-human-Bax, pCMV-Fas, pcDNA3-procaspase-3, or pcDNA3-procaspase-7 and either 9 μg of control plasmid pcDNA3myc or pcDNA3myc-Survivin or pcDNA3myc-XIAP. Transfection efficiency was uniformly ≥50%, as meas-

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[3] The abbreviations used are: IAP, inhibitor of apoptosis protein; BIR, baculovirus IAP repeat; GFP, green fluorescence protein; DAPI, 4′,6-diamidino-2-phenylindole; DEVD, AspGluValAsp; ECL, enhanced chemiluminescence; NCI, National Cancer Institute.

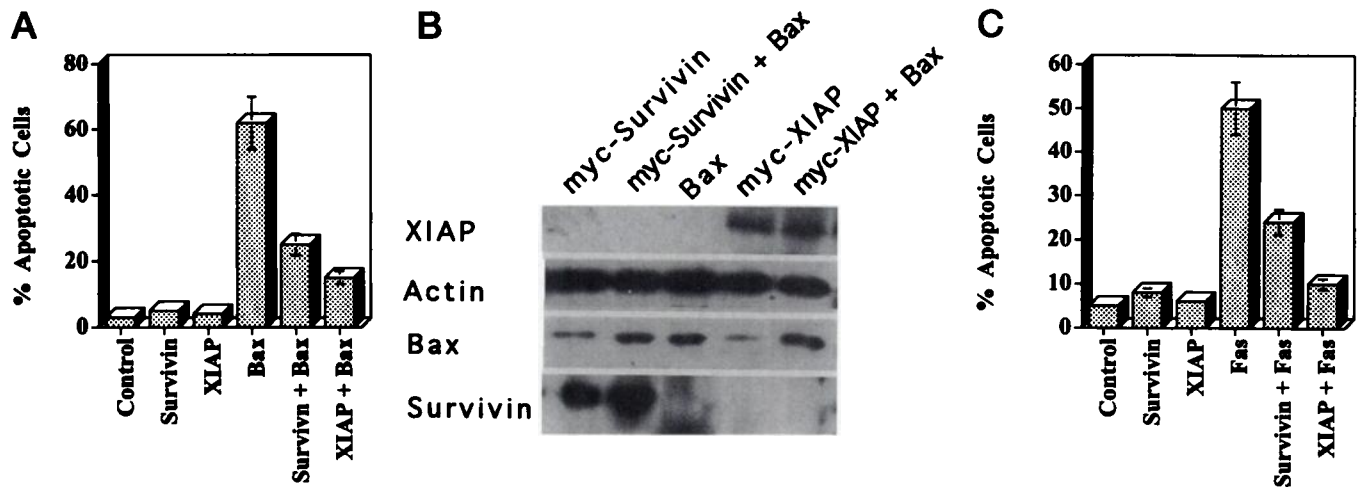

Fig. 1. Survivin inhibits Bax- and Fas-induced apoptosis in 293 cells. *A*, 293 cells were transfected with pcDNA3myc (*control*), pcDNA3myc-Survivin, pcDNA3myc-XIAP, pcDNA3-Bax, pcDNA3myc-Survivin + pcDNA3-Bax, or pcDNA3myc-XIAP + pcDNA3-Bax plasmid DNAs. One day later, the percentage of apoptotic cells (DAPI-staining) was determined (mean ± SE for three determinations). *B*, the expression of the transfected proteins was evaluated by immunoblot assay using 50 μg/lane of total protein. *C*, apoptosis was induced by transfection of pCMVFas, and the percentage of dead cells was determined 1 day later (mean ± SE for three determinations).

ured by cotransfection of the GFP marker plasmid pEGFP (Clontech Laboratories, Inc.). After culturing for 24 h, both the floating and attached cells were harvested, fixed in PBS containing 3.7% formaldehyde for 10 min, and stained with 10 μg/ml DAPI. The percentage of cells with apoptotic nuclei was counted among the GFP-positive cells by fluorescence microscopy. The remaining cell pellets were used for protease assays, immunoblot analysis, or were lysed in CoIP-buffer (10 mM HEPES [pH 7.5], 100 mM KCl, and 0.2% NP40) for protein binding assays. For all experiments, transfection and scoring were performed on randomized coded dishes.

Protein Binding and Immunoblot Assays. Lysates were cleared at 16,000 × *g* for 30 min. Myc-tagged IAP proteins were immunoprecipitated with 10 μl of anti-myc (9E10) antibody immobilized on Protein G-Sepharose (Santa Cruz Biotechnology) for 2 h. Immunoprecipitates were washed three times with CoIP-buffer, and bound proteins were analyzed by SDS-PAGE/immunoblot assay using 750 mM Tris/14% polyacrylamide gels. Immunoblotting for caspases was performed as described after normalizing cell lysates for total protein content. Antisera specific for caspase-3, -7, and -8 were prepared as described (25, 28, 29).

Caspase Activation in Cytosolic Extracts. Cytosolic extracts were prepared from 293 cells as described (30). Briefly, cells were washed with ice-cold buffer A (20 mM HEPES [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT) and suspended in 1 volume of buffer A. Cells were incubated on ice for 20 min and then disrupted by passage through a 26-gauge needle 15 times. Cell extracts were clarified by centrifugation at 16,000 × *g* for 30 min, and the resulting supernatants were stored at −80°C. For initiating caspase activation, either 10 μM horse heart cytochrome *c* (Sigma Chemical Co.) together with 1 mM dATP or 100 nM purified recombinant active caspase-8 was added to extracts (10 mg total protein/ml). Caspase activity was assayed by release of amino-4-trifluoromethyl-coumerin (Enzyme System Products) from DEVD-containing synthetic peptides using continuous-reading instruments as described (31, 32).

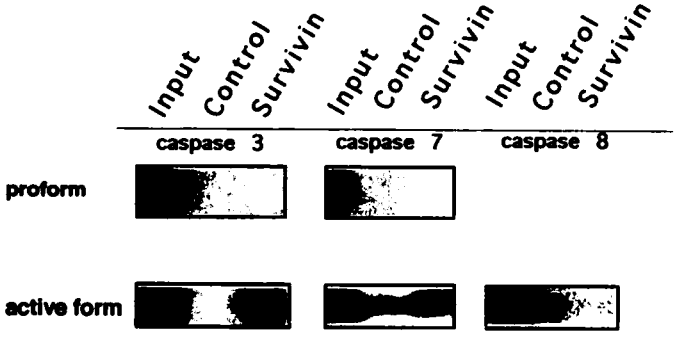

Fig. 2. Survivin binds caspase-3 and -7 *in vitro*. 293 cells were transfected with plasmids encoding myc-Survivin or with pcDNA3myc control plasmid. One day later, cell lysates were prepared for coimmunoprecipitation experiments. IAPs were immunoprecipitated with anti-myc antibody immobilized on protein G-Sepharose and mixed with recombinant active caspase-3, -7, and -8 (*bottom*) or unprocessed (*top*) caspase-3 and -7. Immunoblot analysis with anti-caspase-3, -7, and -8 antibodies was used for detection of immune complexes.

Expression of Survivin in Human Tumor Cell Lines. Lysates from the NCI 60 cell line anticancer drug screening panel were prepared in the presence of protease inhibitors. After normalization for total protein content (50 μg/lane), samples were subjected to immunoblot analysis using a monoclonal anti-Survivin antibody, generously provided by D. Altieri (Yale University School of Medicine, New Haven, CT; 33). Data on X-ray films were quantified by scanning densitometry using the IS-1000 image analysis system (Alpha Innotech Co.). Results from a recombinant Survivin protein-standard included on blots were used to normalize all data before estimating the amount of Survivin protein (ng/50 μg of total protein). Data from two independent Survivin protein standard-containing blots were within 10% agreement.

Results

Survivin Inhibits Bax- and Fas-induced Apoptosis in 293 Cells. Two major pathways for caspase activation have been described. One of these is engaged by tumor necrosis factor receptor-family members such as Fas (CD95) and that involves recruitment to the receptor and activation of proximal caspases, particularly caspase-8 (18, 34). The other pathway involves release of cytochrome *c* from mitochondria, resulting in binding of cytochrome *c* to Apaf-1 in the cytosol, and activation of pro-caspase-9 (17, 18). Both of these pathways converge on downstream effector caspases, particularly caspase-3 and -7 (17, 18).

To explore which apoptosis pathways are blocked by Survivin, transient transfection experiments were performed in human 293 cells using overexpression of the Bax or Fas proteins as stimuli for inducing apoptosis. Bax has been shown to induce release of cytochrome *c* from mitochondria and, thus, was used here as a means of triggering the Apaf-1/caspase-9 pathway (35). Transfection of a Bax-encoding plasmid into 293 cells caused an ~7-fold increase in the percentage of apoptotic cells, as determined by DAPI staining, compared with cells transfected with a control plasmid (Fig. 1*A*). In contrast, when Bax- and Survivin-encoding plasmids were cotransfected into 293 cells, Bax-mediated cell death was reduced to approximately one-third (Student's *t* test, $P < 0.01$) the amount observed with Bax alone. Under the same conditions, XIAP was more potent than Survivin, almost completely inhibiting Bax-induced apoptosis. Immunoblotting experiments confirmed the

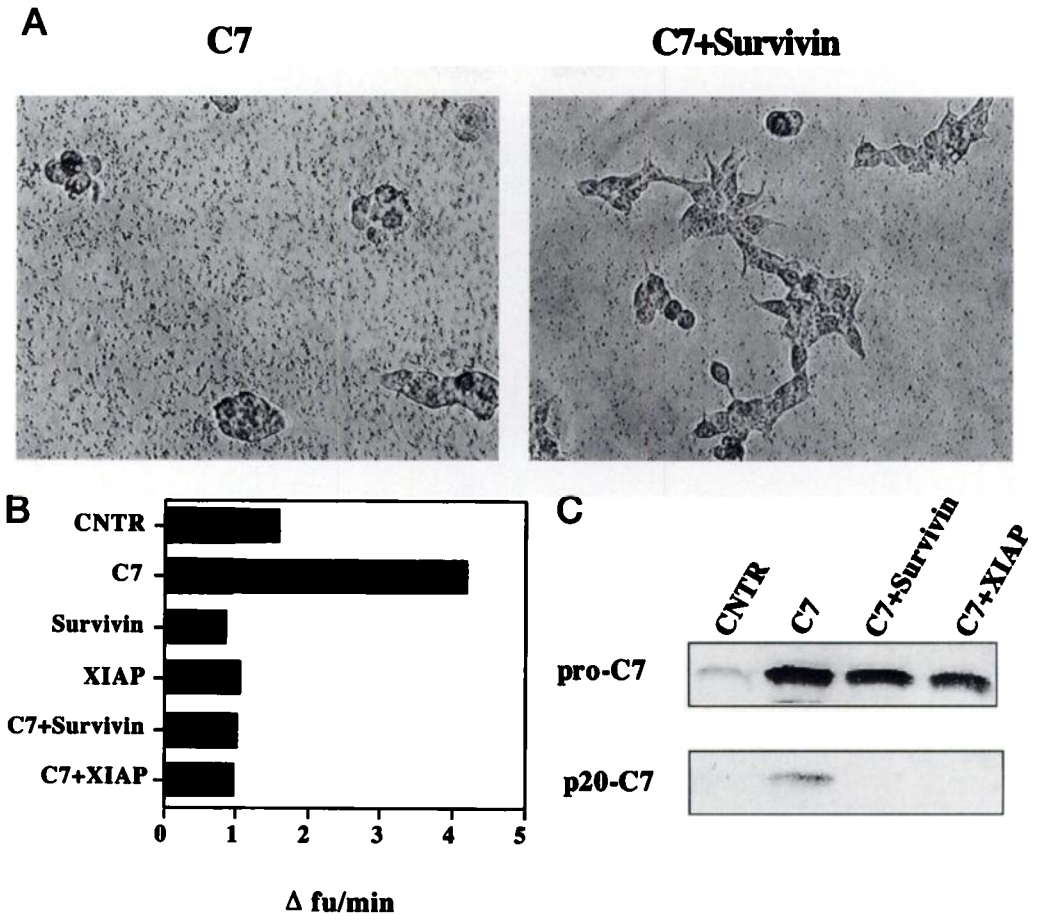

Fig. 3. Survivin protects 293 cells from procaspase-7-induced apoptosis and inhibits processing of procaspase-7. 293 cells were transfected with plasmids encoding control vector, caspase-7, Survivin, XIAP, or various combinations of these as shown. *A*, phase contrast pictures of 293 cells taken at 20 h after transfection with either caspase-7 (*left*) or caspase-7 + Survivin (*right*). *B*, DEVD-AFC cleavage activity was measured using lysates from the transfected 293 cells. Lysates were normalized for total protein content. *C*, cell extracts (50 μg) were subjected to 16% SDS-PAGE. Immunoblot analysis with anti-caspase-7 antibody was used for detection using an ECL-based detection method. The positions of unprocessed and processed caspase-7 are indicated. Similar results were obtained for caspase-3 (data not shown).

expression of the myc-tagged IAP proteins in the expected transfected cells, suggesting that the more complete inhibition of Bax-induced apoptosis was not due to higher levels of myc-XIAP protein compared with myc-Survivin. These immunoblot experiments also verified that Survivin did not inhibit the production of Bax protein in 293 cells (Fig. 1*B*). Similar observations were made when Fas overexpression was used as the apoptotic stimulus, rather than Bax. Coexpression of Survivin with Fas reduced apoptosis by over half, whereas XIAP was more potent (Fig. 1*C*). These experiments suggest that Survivin inhibits events downstream of both Bax and Fas.

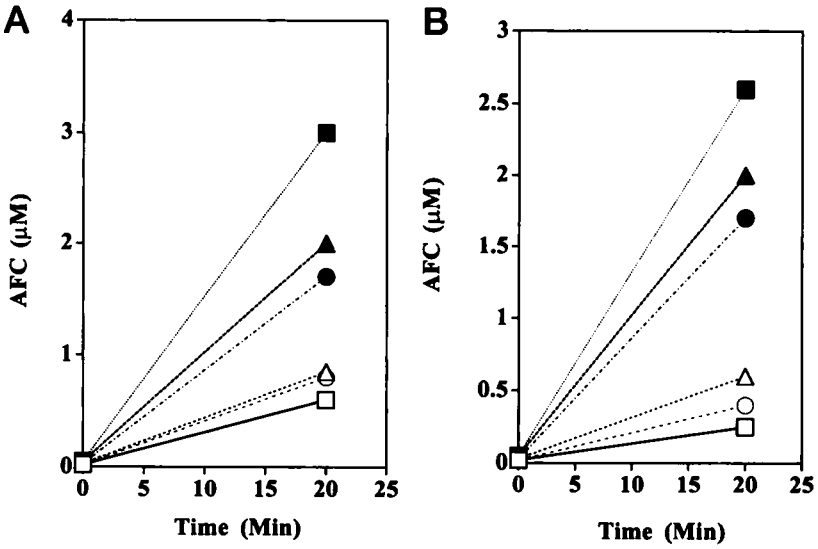

Fig. 4. Survivin inhibits cytochrome *c* induction of DEVD-cleavage activity. *A*, cytochrome *c* (10 μM) and dATP (1 mM) or recombinant active caspase-8 (100 nM; *B*) were added to cytosolic extracts prepared from 293 cells transfected with plasmids encoding myc-Survivin or myc-XIAP or with pcDNA3myc control plasmid. DEVD-specific protease activity was measured using lysates that were normalized for total protein content (11 mg/ml). *A*, □, control (untreated); ■, control (Cyto *c*); △, Survivin; ▲, Survivin + Cyto *c*; ○, XIAP; ●, XIAP + Cyto *c*. *B*, □, control (untreated); ■, control (Casp 8); △, Survivin; ▲, Survivin + Casp 8; ○, XIAP; ●, XIAP + Casp 8.

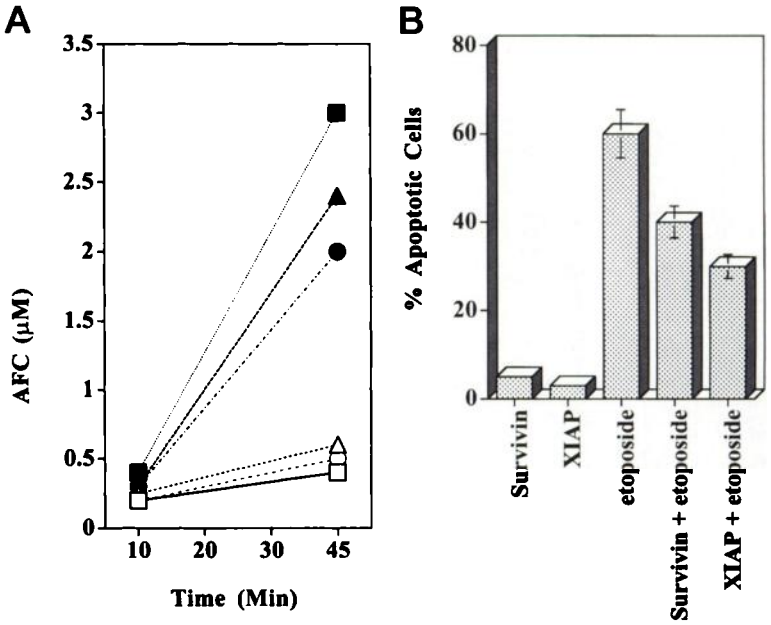

Fig. 5. Survivin inhibits etoposide-induced apoptosis and generation of caspase-like protease activity in cells. 293 cells were used directly or transiently cotransfected with a GFP-marker plasmid (pEGFP) together with either a control plasmid pcDNA3myc or an expression plasmid encoding myc-tagged Survivin or myc-tagged XIAP. After 6 h, etoposide (10 μM) was added to cultures. Lysates were prepared and apoptosis was assessed at 48 h after transfection. Immunoblot analysis confirmed production of comparable amounts of the myc-tagged Survivin protein in each dish (data not shown). *A*, DEVD-AFC hydrolysis was measured using lysates from the transfected 293 cells. Representative progress curves are shown. □, control (untreated); ■, control (etoposide); △, Survivin; ▲, Survivin + etoposide; ○, XIAP; ●, XIAP + etoposide. *B*, the percentage of apoptotic cells was determined by microscopic evaluation of DAPI-stained nuclei in GFP-positive cells. Data represent mean ± SE (n = 3).

Survivin Binds to Active Caspase-3 and -7 *in Vitro*, but not to Their Proforms or to Caspase-8. Because XIAP and some other IAP-family proteins have been shown to bind caspase-3 and -7 (15, 16), we examined the ability of Survivin to bind these cell death proteases *in vitro*. Attempts to produce active Survivin in bacteria proved unsuccessful, apparently due to misfolding as assessed by circular dichorism and nuclear magnetic resonance analysis (data not shown). We, therefore, expressed Survivin in human cells. Using lysates from transiently transfected 293 cells, myc-tagged Survivin was immunoprecipitated with anti-myc antibody, and the resulting immune complexes were incubated with recombinant active caspase-3, -7, and -8 or unprocessed caspase-3 and -7 (Fig. 2). After extensive washing, the resulting immune-complexes were analyzed by immunoblotting using antisera specific for these caspases (25, 28, 29). Active caspase-3 and -7 coimmunoprecipitated with Survivin. In contrast, the inactive proforms of caspase-3 and -7 did not bind Survivin. Similar to previous reports for XIAP and other IAP-family proteins, Survivin also did not bind *in vitro* to active caspase-8. Thus, Survivin binds the active forms of the executioner proteases caspase-3 and -7, but not the upstream initiator caspase-8.

Survivin Inhibits Apoptosis Induced by Overexpression of Procaspase-3 and -7. To further analyze the inhibition of effector caspases by Survivin, we transiently cotransfected 293 cells with either procaspase-3 or -7 and either Survivin or XIAP. Survivin potently inhibited apoptosis induced by overexpression of either procaspase-3 or -7 and also markedly reduced the accumulation of DEVD-cleaving caspase activity in cytosolic extracts derived from these cells (Fig. 3, *A* and *B*, and data not shown). Moreover, cotransfection of Survivin prevented spontaneous processing of caspase-7 (Fig. 3*C*) and caspase-3 (data not shown) to their active forms. Because Survivin binds to the active forms of caspase-3 and -7 *in vitro* but not to their proforms, the most likely explanation for this result is that Survivin prevents active caspase-3 and -7 molecules from cleaving their own proforms (*i.e.*, blocking an amplification mechanism in which a few spontaneously activated caspase-3 or -7 molecules cleave additional procaspase proteins). XIAP exhibited similar effects, suggesting that Survivin and XIAP perform similar or the same functions in cells overexpressing these procaspases. These experiments, therefore, provide further evidence that Survivin directly inhibits caspases.

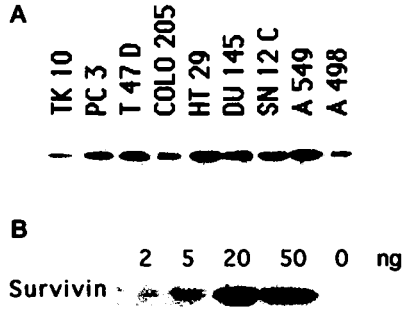

Fig. 6. Examples of Survivin protein expression in human tumor cell lines. *A*, examples of immunoblot data are shown for several human tumor cell lines. In all cases, lysates were normalized for total protein content (50 μg) before SDS-PAGE/immunoblot assay using a mAb against Survivin (33). *B*, An example of a Survivin standard curve is presented, where 0–50 ng of purified Survivin protein was subjected to immunoblot analysis using an ECL-based detection method.

Survivin Inhibits Cytochrome *c*-induced DEVD-Cleavage Activity in Cell Lysates. Addition of cytochrome *c* and dATP to cytosolic extracts prepared from human 293 cells resulted in the rapid accumulation of DEVD-specific protease activity (Fig. 4*A*). Cytochrome *c*-induced DEVD-cleaving activity was substantially reduced in lysates prepared from Survivin- or XIAP-overexpressing 293 cells. Similar results were obtained after the addition of recombinant active caspase-8 (100 nM) to the same lysates (Fig. 4*B*). Thus, Survivin inhibited cytochrome *c*- and caspase-8-induced activation of caspases which cleave the DEVD-tetrapeptide *in vitro*.

Survivin Inhibits Apoptosis and Caspase Activity in Etoposide-treated Cells. The ability of Survivin to suppress cytochrome *c*-induced caspase activity in cell lysates suggested that this inhibitor could also protect intact cells from apoptosis inducing stimuli that cause release of cytochrome *c* from mitochondria. The anticancer drug etoposide inhibits topoisomerase II, resulting in DNA damage and subsequent release of cytochrome *c*, activation of DEVD-cleaving caspases, and apoptosis (36–39). Control- or Survivin-transfected 293 cells were treated with etoposide. The percentage of apoptotic cells and caspase activity in cell extracts were subsequently measured 2 days later. Treatment of control (pcDNA3-myc) transfected 293 cells with 10 μM etoposide resulted in accumulation of DEVD-cleaving activity, whereas little DEVD-cleaving activity was present in lysates

Table 1 *Survivin protein expression in the NCI anticancer drug screening panel of tumor cell lines*

Data represent estimated Survivin protein levels (ng per 50 μg of total protein) based on quantification of immunoblot data by densitometric scanning and extrapolation from a Survivin protein standard curve. Data were scored as follows: 0, undetectable: +/−, 1 to ⇐ 5 ng/50 μg; +, 5 to ⇐ 10 ng/50 μg; ++, 10 to ⇐ 15 ng/50 μg; +++, 15 to ⇐ 20 ng/50 μg; ++++, >20 ng/50 μg. Tumor cell lines are grouped by categories.

Breast		**Leukemia/lymphoma**		**Ovarian**	
BT-549	++	CCRF-CEM	++++	IGROV-1	++++
HS578T	+/−	K562	++	OVCAR-3	+++
MCF-7	+++	MOLT-4	++++	OVCAR-4	++++
MCF7ADR/RES	++++	RPMI-8226	+	SK-OV-3	+/−
MDA-MB-231	+	SR	++		
MDA-MB-435	+				
MDA-N	++			**Prostate**	
T47D	++++			DU-145	++
		Lung		PC-3	++
		A549	++++		
		EKVX	++++		
Colon		HOP-62	+++	**Renal**	
COLO205	++	HOP-92	++++	786-0	++
HCT-15	+	NCI-H322M	+++	A498	+
HCT-116	++	NCI-H226	+++	ACHN	+
HCC-2998	+++	NCI-H23	+	CAKI-1	+/−
HT29	++++	NCI-H522	+++	RXF-393	+/−
KM-12	−/+	NCI-H460	+++	SN12C	++
SW-620	+++			TK-10	+
				UO-31	+/−
CNS		**Melanoma**			
SF-268	+/−	LOX-IMVI	+++		
SF-295	+	MALME-3M	+		
SF-539	+/−	M14	+		
SNB-19	+++	SK-MEL-2	++++		
SNB-75	++++	SK-MEL-28	+		
U251	+++	UACC-62	++++		
		UACC-257	++		

prepared from untreated cells (Fig. 5*A*). In contrast, 293 cells transfected with plasmids encoding myc-tagged Survivin or myc-XIAP accumulated less DEVD-cleaving activity after treatment with etoposide, with XIAP showing greater inhibition than Survivin. These inhibitory effects of Survivin and XIAP overexpression on etoposide-induced caspase activity were paralleled by reductions in apoptosis, as determined by examining the morphology of DAPI-stained 293 nuclei (Fig. 5*B*), with XIAP again providing greater protection than Survivin. Immunoblot assays confirmed expression of myc-XIAP and myc-Survivin proteins, revealing that myc-Survivin was expressed, at least as high as those of myc-XIAP (data not shown).

Survivin Expression in the NCI 60 Tumor Cell Line Panel. The relative levels of Survivin protein were compared by immunoblotting among 60 human tumor cell lines used for the NCI's anticancer drug screening program, using an anti-Survivin monoclonal antibody (33). Representative results are shown in Fig. 6*A*, and the data are summarized in Table 1. An example of a standard curve generated with recombinant Survivin protein is presented, where 0–50 ng of purified Survivin protein was subjected to immunoblot analysis using an ECL-based detection method (Fig. 6*B*). Survivin protein was detected at various levels in all tumor cell lines analyzed. The highest relative levels of Survivin were present in lung and breast cancer cell lines, whereas relatively low levels of Survivin were present in renal cancers.

Discussion

In this study, we show that Survivin is widely expressed in human cancer cell lines and present evidence that it can inhibit the accumulation of caspase activity and suppress apoptosis in cells subjected to diverse apoptotic stimuli.

Activation of effector caspases, such as caspase-3 and -7, is a nearly universal event associated with apoptosis induced by multiple stimuli. These terminal effector caspases account for most of the DEVD-cleaving activity that accumulates in cells undergoing apoptosis (18).[4] Previous studies using recombinant purified XIAP, c-IAP1, and c-IAP2 proteins demonstrated that these IAP-family proteins can directly bind to and potently inhibit active caspase-3 and -7 *in vitro*, whereas little or no binding to the unprocessed proforms of these cell death proteases was observed (15, 16). Although cytosolic lysates from Survivin-overexpressing cells exhibited resistance to cytochrome *c*- and caspase-8-mediated caspase activation, we were unable to produce recombinant Survivin protein in bacteria that displayed bioactivity when added to cytosolic lysates prepared from untransfected cells. Thus, unlike other IAPs, it seems that bacteria-produced Survivin protein may either be misfolded or perhaps lacking posttranslational modifications or cofactors for preservation of its bioactivity.

Nevertheless, though we were prevented from providing direct evidence, the correlative data presented here suggest that Survivin may function similarly to other human IAPs, binding to active caspase-3 and -7 (but not to their zymogens or active caspase-8) and preventing the accumulation of DEVD-cleaving caspases in cytosolic lysates and intact cells treated with agents known to trigger caspase activation and apoptosis. However, compared with XIAP, Survivin was consistently less potent at suppressing generation of DEVD-cleaving caspase activity and apoptosis. Comparisons of Survivin and XIAP binding to caspases *in vitro* using proteins immunoprecipitated from transfected human cells are nonquantitative, but also suggest that XIAP binds more efficiently to caspases than Survivin (data not shown). Taken together, these observations raise the possibility that Survivin is either a somewhat less efficient inhibitor of caspases compared with XIAP or that its activity is regulated in a manner that renders only a fraction of the total Survivin molecules competent to bind and inhibit caspases. In this regard, the structural differences between Survivin and the other human IAPs might serve as a potential molecular basis for variations in caspase recognition and apoptosis suppression. For instance, the lack of a RING-finger in Survivin, even if not directly involved in caspase binding (19), could influence some conformational changes of Survivin compared with other IAPs. Experiments are under way to attempt to resolve these competing models.

The observation that Survivin and several other human IAPs do not bind active caspase-8, but nevertheless inhibit caspase-8-induced activation of DEVD-cleaving enzymes in cytosolic extracts *in vitro*, is

[4] Unpublished observations.

consistent with previous studies (15, 16) that have demonstrated that these antiapoptotic proteins suppress the downstream terminal effector caspases, but not the upstream initiators. Further support for this notion comes from the observation that Survivin and other human IAPs interfere with apoptosis induced not only by caspase-8-activating proteins such as Fas (CD95), but also apoptosis induced by overexpression of Bax, which seems to engage the apoptotic pathway at the level of mitochondria by inducing cytochrome *c* release (35). Cytochrome *c* release results in activation of the CED-4 homologue Apaf-1, initiating an apoptotic protease cascade that begins with processing of caspase-9 and resulting in activation of caspase-3 (30). Thus, the demonstration here that Survivin suppresses caspase activation and apoptosis induced by both Fas/caspase-8 and Bax/cytochrome *c* implies that it functions at a point of convergence of these apoptotic pathways; namely, the terminal effector caspases such as caspase-3 and -7. This interpretation is further underscored by the finding that apoptosis and spontaneous processing of procaspase-3 and -7 are inhibited by Survivin in cells.

Our survey of Survivin protein levels in the NCI's 60 tumor cell line screening panel confirms previous observations derived from primary tumor specimens that indicated that Survivin is commonly expressed in human cancers. However, the relative levels of Survivin protein were variable among the tumor lines evaluated, implying differential regulation of the expression of this antiapoptotic protein within cancers. Although relatively little work has been done thus far to compare the levels of Survivin in tumors with other biomarkers or with clinical outcome, it has been reported that Survivin expression in neuroblastomas correlates with clinically more aggressive, histologically unfavorable disease (40). Moreover, higher levels of Survivin protein as determined by immunostaining and p53 accumulation (indicative of mutant p53) were positively correlated in a survey of gastric cancers (33), implying an association of Survivin with more aggressive disease. The variability in Survivin levels seen here among the NCI's 60 human tumor cell lines suggests that further correlative studies of Survivin expression with clinical outcome and other biomarkers should be informative with regards to assessing the prognostic significance of this apoptotic protein.

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